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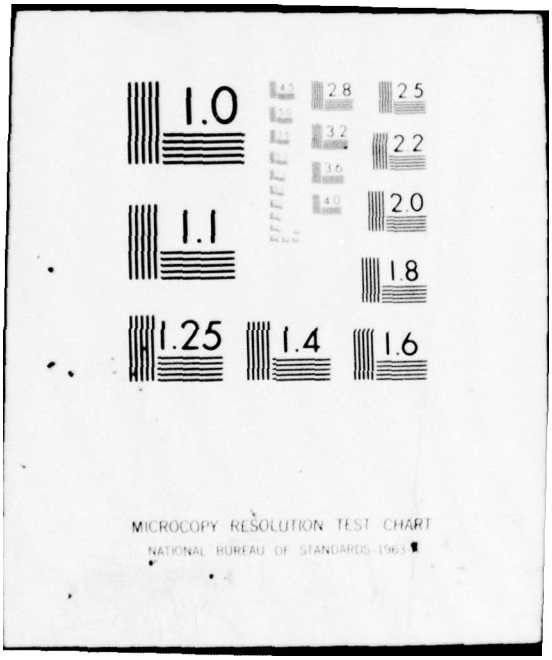
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(6) The Use of a Photodensitometric Technique to Evaluate the Efficacy of Antiviral Compounds against Swine Influenza (Influenza A/New Jersey) Infection in Mice.

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In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on the Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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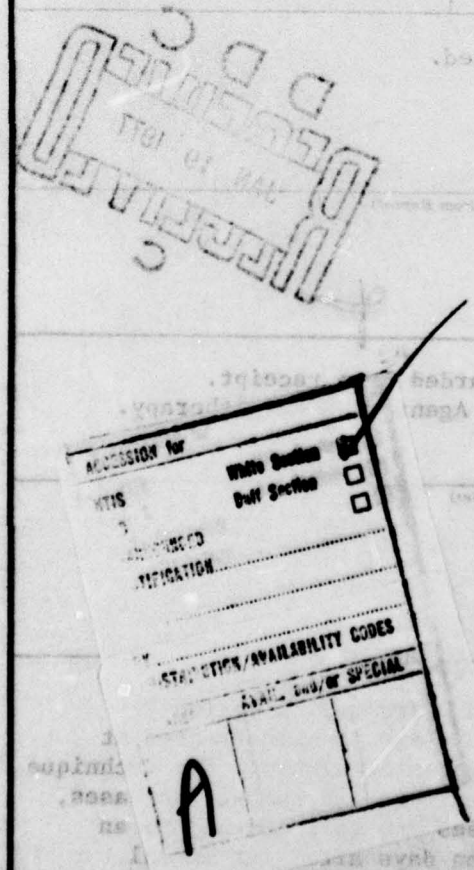
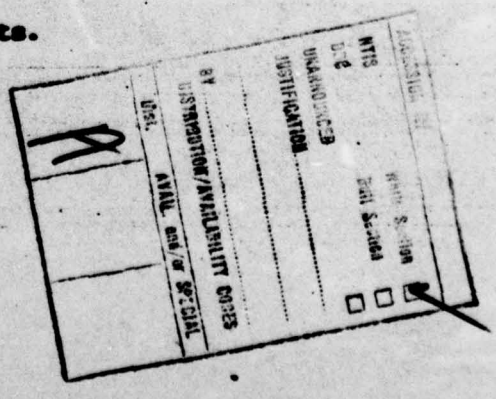
20. Abstract

2% glutaraldehyde at constant pressure. Lungs were processed in a routine manner, sectioned at standard levels, and stained with hematoxylin and eosin. Using standard photomicrography equipment, multiple optical density measurements were made of lung sections in a carefully controlled systematic manner and a mean optical density determined for each lung. The optical density of lungs of mice pretreated with amantadine, rimantadine, or ribavirin was significantly reduced compared with infected-untreated controls ($P < 0.01$). If treatment was delayed until 15 h after infection, amantadine and ribavirin were effective in reducing pulmonary optical density but rimantadine was without effect. These findings correlated well with mean lung weight of each group; however, the sensitivity of the optical density technique was greater. The method offers promise as a reliable means of objectively quantifying the pulmonary response to a variety of infectious, toxic, and therapeutic agents.



ABSTRACT

During studies of swine influenza virus (A/New Jersey) infection a technique was devised to quantify the pulmonary lesions in mice treated at different time intervals with antiviral chemotherapeutic agents. The technique is based on the premise that as the severity of microscopic change increases, the optical density of lung sections also increases due to edema and to an increase in cell numbers in infected lungs. Seven days after intranasal instillation of the virus, mice were killed and the lungs were perfused with 2% glutaraldehyde at constant pressure. Lungs were processed in a routine manner, sectioned at standard levels, and stained with hematoxylin and eosin. Using standard photomicrography equipment, multiple optical density measurements were made of lung sections in a carefully controlled systematic manner and a mean optical density determined for each lung. The optical density of lungs of mice pretreated with amantadine, rimantadine, or ribavirin was significantly reduced compared with infected-untreated controls ($P < 0.01$). If treatment was delayed until 15 h after infection, amantadine and ribavirin were effective in reducing pulmonary optical density but rimantadine was without effect. These findings correlated well with mean lung weight of each group; however, the sensitivity of the optical density technique was greater. The method offers promise as a reliable means of objectively quantifying the pulmonary response to a variety of infectious, toxic, and therapeutic agents.



Animal models for evaluation of potential antiviral compounds have frequently been developed using human disease pathogens adapted to produce high mortality. The efficacy of the compounds is thus measured by survival rate of treated infected animals compared with untreated controls. These model systems are unnatural and unrealistic when the purpose intended for the drug is to treat diseases of man and animals which are characterized by high morbidity and low mortality (6). During studies of swine influenza (influenza A/New Jersey), (Scott, G. H., manuscript in preparation), it was observed that the mouse-adapted virus did not have lethal virulence for adult mice and thus resembled natural influenza infections of man (4). It was necessary to seek another end-point to measure the effectiveness of antiviral compounds on the respiratory disease in mice.

Numerous systems have been proposed to grade morphologic changes occurring in the lungs of animals infected with various respiratory disease-producing agents. Many are not completely objective. The majority of the reliable procedures are morphometric where various measurements can be made of pulmonary structure to obtain consistent quantitative values (2). These techniques require considerable time to perform and thus are inefficient when numerous specimens must be examined.

A technique, described for studying emphysema in pigs and rats, was based on the postulate that emphysematous lung sections are less optically dense than normal lung and that differences could be quantified by a densitometer (3). A similar procedure has been employed to determine degree of erythrocyte aggregation in vitro (1). Likewise, pulmonary disease characterized by edema and cell infiltration into

alveolar spaces and the interstitium should increase optical density. If this premise were correct, the effect of drugs, toxins, or chemicals, on the pulmonary response could be quantitatively assessed and compared in an unbiased, completely objective manner. This technique was utilized to compare the efficacy of antiviral chemotherapeutic agents in mice infected with swine influenza virus.

MATERIALS AND METHODS

Virus. Influenza, A/NJ/8/76 virus, ($H_{sw}1N1$), was passed six times in embryonated eggs; adaptation to mice was accomplished in nine serial passages by intranasal (i.n.) instillation of supernatant from a suspension of homogenized lungs from mice infected 3 to 4 days previously. After the 9th passage, allantoic cavities of 10-day-old embryonated eggs were inoculated with supernatant from infected lungs. After incubation for 48 h at 35 C, infected allantoic fluid was harvested and clarified by centrifugation at 1200 x g at 4 C for 15 min. Antibiotics were added to the clarified fluid to achieve a final concentration of 250 units of penicillin/ml and 100 µg of streptomycin/ml; aliquots of the suspension were stored frozen at -60 C. The titer of the infected allantoic fluid was $10^{7.7}$ median egg infectious doses (EID_{50}) per ml.

Experimental design. Eight week old (25 g) outbred, female Swiss mice [Crl:COBS CD-I (ICR) BR] obtained from the Sendai-free colony of Charles River Laboratories were divided into eight groups each containing 10 mice. Three groups were prophylactically treated with amantadine, rimantadine or ribavirin in the drinking water at a dosage of 0.25 mg/ml (approximately 60 mg/kg/mouse/day) 24 h prior to intranasal instillation of $10^{6.1}$ EID_{50} of influenza virus in 0.05 ml. Three other groups were treated therapeutically with each of the drugs beginning 15 h after virus inoculation. In all groups, treatment was then continued for 7 days after inoculation of the virus. Two control groups of mice were utilized, one was infected but not treated and the other was neither infected nor treated.

Necropsy procedures. Seven days after infection, all mice were killed by cervical dislocation. In half the mice from each group, lungs were dissected from surrounding tissue and weighed to the nearest milligram. In the remaining mice, the cutaneous tissue of the neck was dissected free from the trachea, and the thorax was opened. A 21-guage hypodermic needle attached to a flask containing 2% glutaraldehyde, pH 7.2, was inserted into the trachea and the lungs were perfused in situ for 30 sec. The pressure of the perfusate was 20 cm of water. To maintain pulmonary distension a small vascular clamp was placed on the trachea. Lungs were then dissected free and suspended in 2% glutaraldehyde.

Perfused lungs were subsequently fixed in 10% buffered neutral formalin and embedded whole in paraffin. Paraffin blocks were sectioned horizontally through the lung lobes and three 6 μm sections were taken at 1.5 mm intervals (Fig. 1). The sections were deparaffinized and stained with hematoxylin and eosin (5). Staining was controlled so that variation was not observed among slides.

Densitometry. The sections of lung were placed on the mechanical stage of a Zeiss Ultraphot II (Carl Zeiss, Oberkochen/Wuerttemberg, Germany) and voltage was adjusted to 15: a filter with peak absorption at 580 and 645 nm was placed between the light source and specimen, and the image was projected onto a viewing screen using the 2.5 X objective. Total magnification was 27 X. Twenty to 35 measurements were made on each section directly from the same location on the viewing screen using a photodensitometer (Model 501 M, Photovolt Corp., New York, NY). Optical densities were measured through 0.5 mm diameter holes spaced 0.85 mm apart in a rectangle of brass foil which was placed on the microscope slide (Fig. 2). The photovolt meter was adjusted to 0

optical density at a place on the slide where there was no tissue. The lung at each perforation was viewed independently for artifacts or extraneous tissue and, if present, the field was eliminated from the sample. Essentially, only alveoli and small bronchioles were measured. The above procedure was repeated for each of the three lung sections and the mean optical density of each lung was calculated.

Statistics. Data on lung weight and optical density were analyzed by one way analysis of variance using the least significant difference test to determine differences between groups. A linear regression analysis was used to determine correlation of mean optical density and mean lung weight of each group (7).

RESULTS

Lesions of swine influenza in untreated infected controls consisted of multifocal areas of bronchiolitis surrounded by thickening of alveoli, minimal to moderate inflammatory cell infiltration, and edema in alveolar spaces. In contrast, lungs of uninfected control mice were free of lesions. These morphologic changes were reflected by an almost 3-fold increase in pulmonary optical density in the untreated infected control group compared to the unifected controls (Table 1, Fig. 3). Consistent readings were obtained by repeating the procedure. In general, with the exception of mice treated with rimantadine 15 h after infection, the mean optical density of treated mice was midway between the values of uninfected and infected-untreated controls and differed significantly from the latter ($P < 0.01$). By analyzing lung weights of the various groups, there was significant reduction of lung weight only for mice given rimantadine prior to virus inoculation and mice give ribavirin 15 h subsequent to infection compared with infected-untreated controls ($P < 0.01$). However, the trend was the same as that observed by densitometry, with the lung weights of infected-treated animals falling between values observed for both control groups. The linear regression analysis of the means of pulmonary optical density against means of lung weight gave a coefficient of correlation of 0.92.

DISCUSSION

This photodensitometric technique provided an excellent means of quantifying the effects of antiviral agents on the pulmonary lesions of mice infected by swine influenza virus. The virus used in this study was characterized by low mortality for adult Swiss mice; thus the infection was similar to many naturally-occurring influenza outbreaks (4). In all likelihood, evaluation of the efficacy of the chemotherapeutic agents was performed under conditions simulating the natural disease. It is important to note that in these studies, lungs from all mice were sampled at the same time after infection when the pneumonia was at a similar stage. Thus, differences in optical density were a reflection of differences in severity of disease rather than qualitative differences in inflammation.

With influenza A/New Jersey infection, good correlation between mean optical density and mean lung weight for the various groups ($r = 0.92$) suggests that both methods have merit in the assessment of antiviral activity. The advantage of the optical density technique over lung weight in this experiment is the increased sensitivity observed with the former method. Reasons for this are: 1) The variability of optical density measurements within groups was less than lung weight; 2) optical density measurements were independent of lung size; and 3) structures not contributing to the pathologic process such as blood, large airways, tissues artifacts, etc. were selectively eliminated by viewing each field prior to obtaining an optical density measurement. In addition, this technique utilized photomicrography equipment already found in most laboratories, thus precluding the expense of more elaborate apparatus.

We feel this technique offers promise as a means of evaluating the response of lungs to a variety of infectious and toxic agents and will provide an objective, unbiased measure of the therapeutic value of various drugs for treating various respiratory diseases.

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TABLE 1. Prophylactic and therapeutic effects of amantadine, rimantadine, or ribavirin on lung optical density and weight in influenza A/New Jersey-infected mice.

Group	Mean value ± SEM	
	Optical density X 10^{-3}	Lung weight (mg)
Prophylaxis + Therapy		
Amantadine	69 ± 4[a,b]	310 ± 14[b]
Rimantadine	68 + 5[a,b]	272 ± 17[a,b]
Ribavirin	71 ± 8[a,b]	332 ± 42[b]
Therapy		
Amantadine	63 ± 2[a]	324 ± 24[b]
Rimantadine	84 ± 11[b]	352 ± 17[b]
Ribavirin	56 ± 3[a]	266 ± 19[a,b]
Controls		
Infected-untreated	98 ± 11[c]	390 ± 36[c]
Uninfected	38 ± 1[c]	142 ± 2[c]

[a]Differs significantly from infected-untreated control ($P < 0.01$).

[b]Differs significantly from uninfected control ($P < 0.01$).

[c]Controls differ significantly from each other ($P < 0.01$).

FIGURE LEGENDS

FIG. 1. Schematic drawing of lateral view of mouse lung showing the level of sectioning.

FIG. 2. Perforated brass cover on microscope slide through which light was transmitted for optical density measurements.

FIG. 3. Effect of antiviral agents on lungs of mice infected with swine influenza.